## Chapter 7

# A primer for lignocellulose biochemical conversion to fuel ethanol

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## INTRODUCTION

Developing a commercial industry for using lignocellulose feedstocks to produce fuel ethanol has taken on greater urgency with increasing concerns regarding US dependency upon imported oil. The US president has repeatedly referred to the need for cellulosic ethanol, the popular press has often touted its advantages (usually over corn ethanol), and in spring 2007 the federal government announced it will support six commercialisation efforts up to a grand total of US$385 million (US Department of Energy, 2007a). The final amounts granted will depend upon completion of milestones. This review will emphasise the available technology and remaining obstacles to realising lignocellulose as a commercial feedstock for ethanol production.

First, it is appropriate to ask: Is lignocellulose needed as a feedstock for ethanol? After all, the US produced over 4.8 billion gallons of grain ethanol in 2006 (Renewable Fuels Association, 2007) and is expected to exceed 12 billion gallons per year by 2015 (Westcott, 2007). However, even 12 billion gallons per year is less than 10% of the current US gasoline usage. Clearly, further options (including conservation) are needed to meet our future oil needs, and lignocellulose is the only renewable feedstock that rivals corn in quantity.

Recently, a joint panel of United States Department of Agriculture (USDA) and Department of Energy (DOE) experts met to estimate the amount of fibrous biomass available for ethanol production. The panel predicted that by 2030 enough biomass could be produced, collected and converted to biofuels to meet up to 20% of the US transportation fuel needs (on an energy basis) (Perlack *et al.*, 2005). Meeting this challenge would consume approximately one billion dry tons of biomass feedstock annually, which would include, in addition to grains, agricultural and forest residues and perennial energy crops.

Finally, including lignocellulose as a feedstock will benefit the entire industry. It will lower the net greenhouse gas emissions (Farrell *et al.*, 2006), expand production without further increasing cost pressure on food and feed markets, extend crop production to land unsuitable for row crops and continue ethanol-related development of rural economies.

## PRODUCTION, STORAGE AND TRANSPORT

Feedstock will be regional and strictly based upon a winning combination of cost (US$40–60 per ton or less) and quantity (Aden *et al.*, 2002). Available biomass is generally taken as that harvested within 50 miles of the ethanol production facility, which means that, even assuming a high ethanol yield (80

gallons per ton, dry matter), a 40-million-gallon-capacity plant will require one half million tons per year. Availability and concentration of biomass will determine where biomass ethanol plants are sited even more so than for corn ethanol.

Available feedstocks are broadly categorised as municipal solid waste (e.g. paper), agricultural residues, forest product-related waste and perennial energy crops cultivated on Conservation Reserve Pasture (CRP) or marginal row crop land (Figure 1). The first offers the advantage of high cellulose contents combined with low lignin and the hope of a tipping fee. Agricultural residues are primarily corn stover ($75\times10^6$ tons per year) and wheat straw ($11\times10^6$ tons per year) but could include any other straw that can be collected in sufficient quantities. Abengoa Bioenergy, Iogen Corp. and POET (formerly Broin) have all announced plans to build production facilities for converting corn stover, mostly cobs, to ethanol. Forest product waste includes wood made available from logging and direct residues from the pulp and paper industry ($134\times10^6$ tons per year). The large paper and pulp company, Weyerhaeuser, has recently announced interest in researching ethanol production, and Mascoma Corp. has received a grant from the State of New York to build a plant for converting wood chips to ethanol. Perennial energy crops include C4 grasses or fast-growing trees such as poplar hybrids. Grasses most often mentioned are miscanthus, switchgrass and reed canary grass (Lewandowski *et al.*, 2003). Ideal traits for these crops are their high yields (greater than two tons, dry matter, per acre), low water and nitrogen requirements and developed agronomics. Many companies view these crops as the next step after exploitation of agricultural residues as feedstocks. While dedicated energy crops have a disadvantage compared to agriculture residues with no coproduct (e.g. corn and wheat kernels), there is the possibility of specifically breeding or genetically engineering these crops for higher conversion yields or less-expensive processing (e.g. *in situ* expression of hydrolytic enzymes). This can also be done for conventional crops such as corn stover, but any introduced trait must not interfere with grain production. Major research efforts of this type are currently underway at Ceres, Inc., in cooperation with the Samuel Roberts Noble Foundation, the Agricultural Research Service and the DOE, the latter announcing they will invest up to US$375 million in three new Bioenergy Research Centers that will be located in Oak Ridge, Tennessee; Madison, Wisconsin; and near Berkeley, California (US Department of Energy, 2007).

Supplying ethanol facilities with herbaceous biomass will require an entirely new infrastructure devoted to harvesting and handling. Corn stover harvesting is now a multistep process that involves cutting and shredding, field drying, windrowing, baling and hauling (Sokhansanj *et al.*, 2002). Shinners *et al.* (2007) examined harvesting and baling corn stover in Wisconsin. They were only able to collect 37% of the available corn stover biomass, and DM (dry matter) losses were 18.1% for bales stored outside over

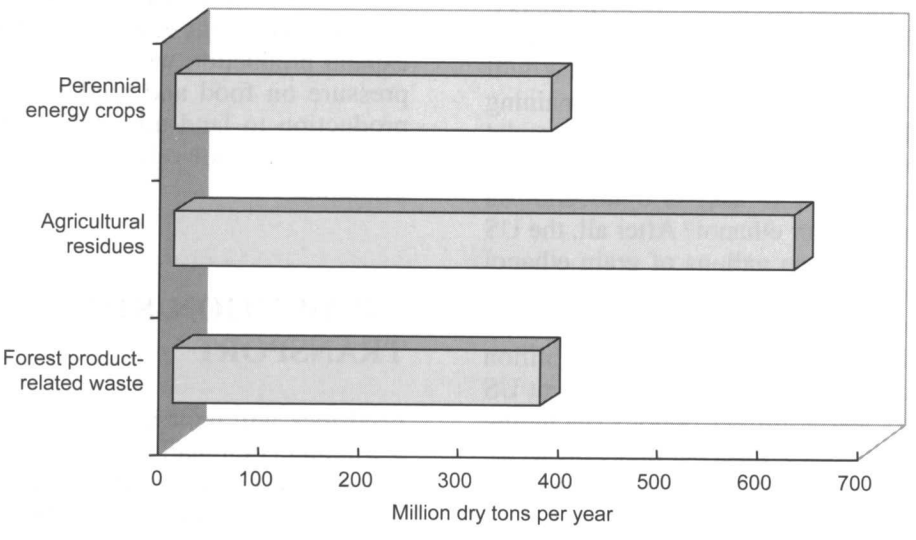


**Figure 1.** US land can supply 1.3 billion tons of biomass for biofuels and still meet other needs (adapted from Perlack *et al.*, 2005).

eight months – losses were only 3.3% for bales stored inside. Single-pass systems are also being explored for corn stover using a modified forage harvester that separates the corn grain and stover (Shinners *et al.*, 2005); however, the stover may need to be ensiled because this technology precludes field drying. Ensiling has also been recommended for avoiding DM losses (Shinners *et al.*, 2007). One-pass system harvesting is more attractive because it saves time, money and energy and avoids further compaction of the soil from multiple passes.

Perennial grasses used as energy crops can be left in the field to dry and even harvested with a one-pass forage harvester (if available). In one study, Sanderson *et al.* (1997) harvested switchgrass in August and November using a multipass system and stored the bales outside. DM losses were 13% for the switchgrass harvested in August and only 5% for the biomass harvested later. They do not report harvest efficiency, but their total yield was a respectable 3.7 tons per acre.

Low bulk density is another challenge confronting the use of herbaceous feedstocks. Shinners *et al.* (2007) calculated an average bale density of 123 kg per cubic metre for their harvested stover. By comparison, shelled dent corn has a bulk density of 721 kg per cubic metre. Straws can be further compacted (up to ten times over that of chopped), but only after added cost and energy. Figure 2 shows how to estimate the differences in storage volume required for these two materials. Besides volume, another difference is that while corn can be stored safely in bins, bales need to be stored in a less convenient manner to minimise their being a fire hazard. Researchers are investigating on-farm pretreatments in hopes of helping farmers realise better price for their biomass. Many of these either involve ensiling or are reminiscent of well-developed forage treatment systems (Sundstol and Owen, 1984).

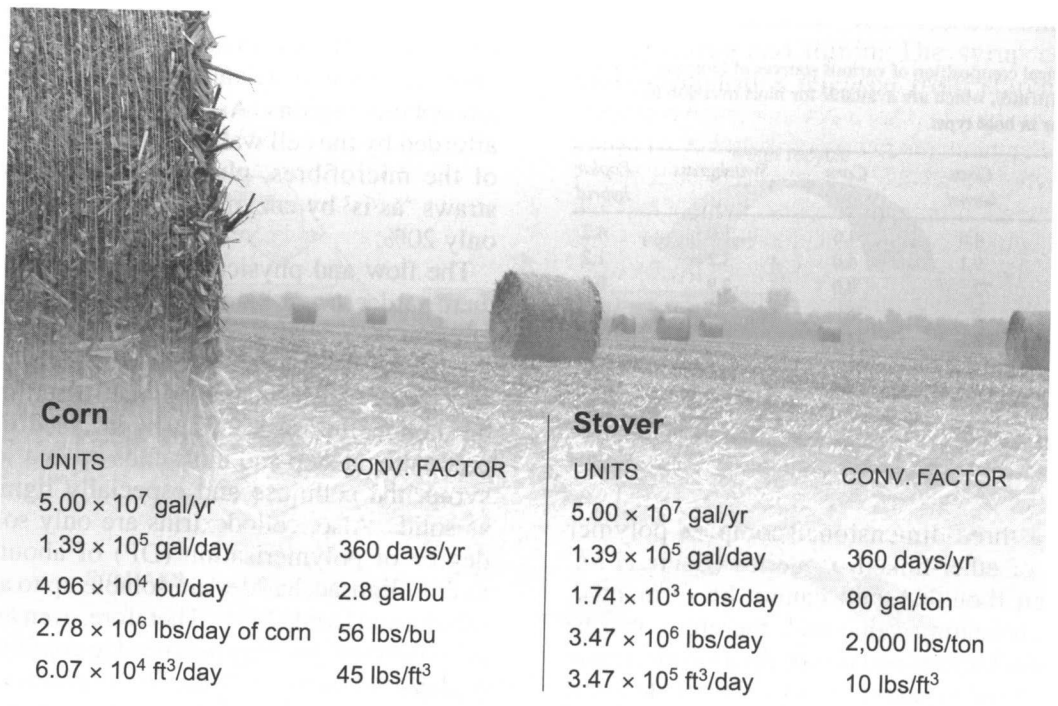


**Figure 2.** Calculations for comparing the volume of storage space required for a day's supply of corn with a day's supply of stover for a 50-million-gallon-per-year plant. A plant producing 50 million gallons per year of ethanol from corn stover would require approximately 4,000 round bales per day, which would occupy 5.7 times the volume of the equivalent amount of corn (1,000 lbs per bale at 15% moisture and 80 gallons of ethanol per ton) (photo courtesy of ARS).

## STRUCTURE AND CHEMICAL COMPOSITION

Plants broadly consist of ether extractables, proteins, carbohydrates, lignin and ash (Table 1). Extractables include waxes and lipids as well as other water-insoluble materials. Carbohydrates are categorised as soluble sugars (sucrose, glucose and fructose), storage carbohydrates (starch and fructans) and structural carbohydrates (cellulose, hemicellulose and pectin). C4 warm-season grasses, which include the major grains, switchgrass and miscanthus, contain minor amounts of pectin, and the amount of soluble sugars and nongrain starch depends upon the maturity at harvest and storage conditions. However, the majority of nongrain carbohydrate is in the form of cellulose and hemicellulose. Grass hemicellulose is composed largely of xylose, which is defined as a pentose sugar because it has five carbons. Cellulose is composed solely of glucose, which is a hexose. Only carbohydrates can be biologically converted into ethanol.

**Table 1. Chemical composition of various sources of biomass (% w/w, DM). Carbohydrates, which are available for bioconversion to ethanol, appear in bold type.**

| *Composition* | *Corn kernel* | *Corn stover* | *Switchgrass* | *Poplar hybrid* |
|---|---|---|---|---|
| Ether Ext. | 4.6 | 4.6 | 1.0 | 4.2 |
| Protein | 9.1 | 4.0 | 3.2 | 1.2 |
| **Starch** | **72** | **0.0** | **3.9** | **0.0** |
| **Cellulose** | **2** | **36** | **28.3** | **42.4** |
| **Hemicellulose** | **3.6** | **23.4** | **24.5** | **19** |
| Klason lignin | Trace | 18.6 | 15.4 | 25.7 |
| Ash | 1.5 | 12.5 | 5.4 | 1.8 |

Dien *et al.* (2006) and Biomass Feedstock Composition and Property Database (http://www1.eere.energy.gov/biomass/feedstock_databases.html).

Lignin is a three-dimensional complex polymer comprised of ether-linked phenolics (Bacic *et al.*, 1988). Even though lignin cannot be fermented, it has a high heating value and, therefore, can be burned to provide heat and power. As an aside, lignin can be converted to liquid fuels by thermochemical processing. Ash includes all the minerals present in the plant. Grasses have more ash than woody material, especially silicon.

Releasing and fermenting the carbohydrates from plant fibres are more difficult than from grains and utilise different processing methods. These arise from differences in structure between storage starch and cell wall fibres, the insoluble nature of the fibres, the rigidity of cellulose versus starch, and wider varieties of carbohydrates present in fibre. Plant fibres are composed of cell walls (Reid, 1997; Bidlack *et al.*, 1992; Somerville *et al.*, 2004). The cell walls support the plant, form vessels to transport water and nutrients and help protect it from pathogens. Plant cell walls have been compared to reinforced concrete, where the hemicellulose serves as concrete filler, cellulose microfibres as reinforcement bars and lignin as the sealant. This complex structure naturally makes cell walls highly resistant to the actions of hydrolytic enzymes.

The rigid structure of cellulose compared to starch makes it much harder to activate for enzymatic digestion. Like starch, cellulose is a homopolymer of glucose (Reid, 1997). However, in cellulose the glucose molecules are joined to form flat linear chains of up to 15,000 units long. Numerous hydroxyl groups and the flatness of the chains allow multiple hydrogen bonds to form with other hydroxyl groups within the same strand and adjacent strands. As a result, individual chains aggregate into much larger microfibres with diameters of 2–10 nm. These fibres are highly crystalline and interspersed with amorphous regions. As a result of the protection afforded by the cell wall structure and compactness of the microfibres, glucose yields from treating straws 'as is' by enzymes are often on the order of only 20%.

The flow and physical properties of fibres make them harder to process as wet flow streams compared to ground grains. Unlike starch, which can be liquefied, even heated fibres remain highly insoluble, and fibre suspensions are prone to settling. While the hemicellulose is often hydrolysed during the pretreatment step and most ends up in a solubilised syrup, the cellulose and especially lignin remain as solids. Also, cellodextrins are only soluble to a degree of polymerisation (DP) of about 6, while malto-oligosaccharides are soluble up to a DP of 60 (Zhang and Lynd, 2004). Therefore, even as cellulose is hydrolysed, the enzymes need to continually cross liquid/solid interfaces – this is one of several reasons hypothesised for why cellulases have rates 100 times slower than amylases (Zhang and Lynd, 2004). Another major reason is the lack of branches, which means fewer ends for the enzymes to attack.

The fourth difference is related to the xylan, which contains xylose and another pentose, L-arabinose. Neither sugar is fermented by *Saccharomyces*

*cerevisiae*. As xylose often accounts for more than 30% of the available carbohydrates in biomass feedstocks, it cannot be ignored. Therefore, biological conversion of lignocellulose has come to rely on genetically modified organisms (GMOs) engineered to ferment pentoses to ethanol in mixed sugar hydrolysates.

## MODEL PROCESS FOR CONVERTING BIOMASS TO ETHANOL

As benefits a nascent industry, there are many ideas being championed as the best process for converting fibrous biomass to ethanol. The most well-characterised process is dilute acid pretreatment coupled with simultaneous saccharification and fermentation (SSF), first conceptualised by Takagi *et al.* (1977) and later developed as the Gulf Process (Katzen *et al.*, 1999; Emert and Katzen, 1980; Blotkamp *et al.*, 1981). SSF has subsequently been refined by the National Renewable Energy Laboratory (NREL) as well as others (Aden *et al.*, 2002). Reviewing this process (Figure 3) is instructive as an introduction to the basic unit operations needed for processing fibrous biomass. After arriving at the facility, biomass is cleaned of foreign objects and milled for size reduction (Mani *et al.*, 2004). It is next pretreated. Pretreatment consists of mixing the solids with a dilute sulphuric acid solution to bring the pH down to 1.0 and heating the acidified biomass briefly in a steam explosion reactor. A steam explosion reactor is commonly used by the pulp and paper industry to remove lignin (Saddler *et al.*, 1993). As its name implies, the reactor consists of a small chamber where high-pressure steam is mixed with the biomass followed shortly by explosive decompression. Steam explosion reactors have the advantages of allowing for rapid steam heating and evaporative cooling of high-solids streams in a continuous manner. As an example, typical reaction conditions for pretreating corn stover are pH 1 and 190°C for 60 seconds (Schell *et al.*, 2003).

The pretreated material is pressed and washed prior to fermentation. The washed solids contain the cellulose and lignin. The syrup consists of monosaccharides released from starch (a minor

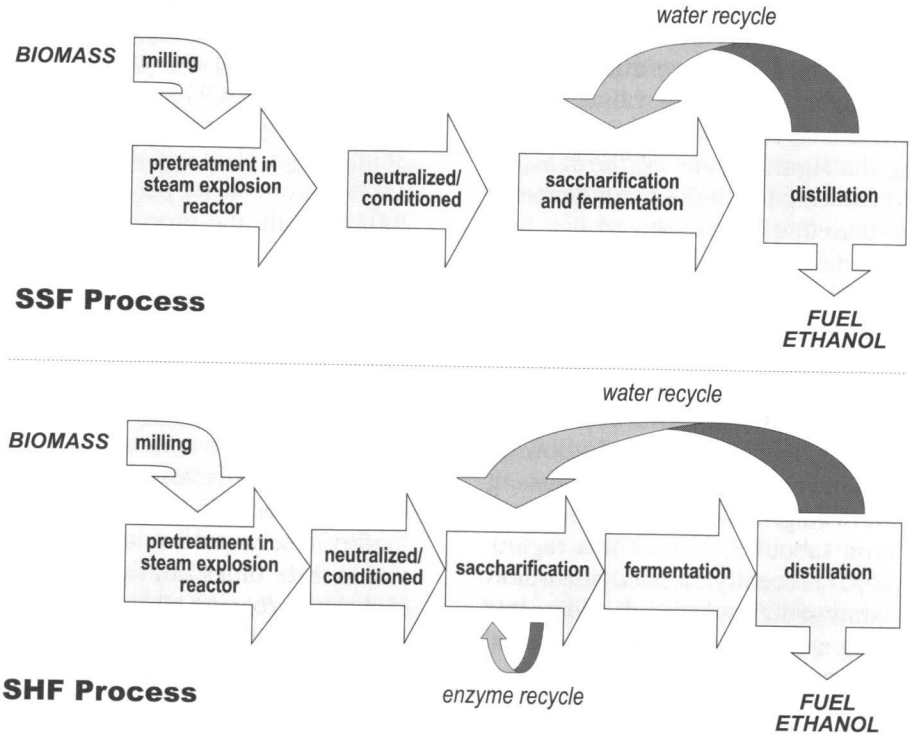


**Figure 3.** Schematics of proposed processes for converting biomass into ethanol.

component of fibrous biomass) and hemicellulose, as well as extractables and acid-digestible lignin. The syrup is neutralised with lime and further processed to remove or neutralise organic by-products formed during the harsh pretreatment that may interfere with the subsequent fermentation. The processed syrup is remixed with the solids and any available recycled process water before entering the bioreactor. Hydrolytic enzymes (e.g. cellulases) and any required fermentation nutrients are added to the bioreactor, and the mash is allowed to ferment for anywhere from 72 to 144 hours. It is generally assumed that a GMO will be used as the catalyst – one that can ferment xylose and possibly arabinose in addition to glucose and other hexoses present. As the enzymes release glucose from the cellulose, the chosen microbe ferments it immediately to ethanol. It is this simultaneous release (saccharification) and fermentation of glucose that is termed SSF. Running in SSF is advantageous because hydrolytic enzymes used to digest cellulose are inhibited when too much glucose and cellobiose accumulates. Keeping their concentrations low greatly increases enzyme efficiency. It also helps to prevent contamination and, provided the glucose concentration is low enough, it may also allow for co-utilisation of glucose and sugars released from hemicellulose. Following fermentation, the residual solids are separated from the beer and the beer is distilled to ethanol. The solids, which contain high heating value lignin, are burned to generate steam and power to run the process. Burning the lignin is one of the reasons ethanol produced from lignocellulose can generate more 'net energy' than that from corn.

Higher solids can be fermented in the bioreactor – thereby increasing the final ethanol concentration – if the solids are partially hydrolysed with enzymes prior to entering the bioreactors. Therefore, it is not uncommon to include a partial hydrolysis step prior to inoculating (sometimes referred to as a hybrid process). This also has the advantage of allowing the enzymes to begin hydrolysing the biomass at a higher temperature (e.g. 50–60°C) than suitable for the fermentation (about 35°C). In this regard, Jorgensen *et al.* (2007) recently blended hot-water-pretreated wheat straw at 40% w/w solids and treated with cellulases using a custom-designed horizontal paddle mixer. They observed that in as little as four hours the mixture changed from a slightly moist solid to a 'very thick paste'. When the undiluted slurry was later fermented with *S. cerevisiae* in an SSF, the overall yield was only about 40% at this very high solids concentration. Alternately, the solids could be completely saccharified prior to fermenting to ethanol (Figure 3). This would have further advantage of removing nonfermentables from the bioreactor. However, running a separate hydrolysis and fermentation (SHF) would depend upon using enough enzymes to compensate for the slow hydrolysis rates incurred at higher glucose concentrations. The Iogen process consists of steam pretreatment followed by SHF.

A variation on SSF is to have the microorganism(s) produce its own enzymes for saccharifying the pretreated biomass. This process is termed consolidated bioprocessing (CBP) (Lynd *et al.*, 2005). The obvious and most direct advantage is to eliminate, or at least greatly reduce, enzyme costs. Examples of this strategy are to genetically engineer *Saccharomyces* to produce cellulases (Den Haan *et al.*, 2007a; Den Haan *et al.*, 2007b; Fujita *et al.*, 2004), taking thermophilic gram-positive bacteria that naturally produce their own hydrolytic enzymes and engineering them to selectively produce ethanol (Demain *et al.*, 2005), or to supplement the native ability of ethanologenic *Klebsiella oxytoca* to use short oligomers by engineering it to also produce cellulase proteins (Wood and Ingram, 1992). Some proponents of CBP have referred it as 'second generation' cellulosic ethanol technology because of the need for further research.

The NREL-DOE Laboratory recently published an extremely detailed techno-economical model simulating a plant capable of converting 2,000 ton (DM) of corn stover per day to ethanol (Aden *et al.*, 2002). While the process parameters and reduced enzyme costs are beyond what is currently feasible, the study is useful in providing an overview of the dilute acid process and insight into cost sensitivities. The target yield cited in this study is approximately 90 gallons of ethanol per ton of dried corn stover with overall conversion yields of 85% for cellulose and 77% for xylose to ethanol.

The above-detailed description shows that bioconversion of lignocellulose is technically feasible. Unfortunately, production costs are estimated to be two to three times and capital costs four to five times higher than for corn ethanol (Wallace *et al.*, 2005). The NREL corn stover model cited above also shows how costs are distributed (Figure 4). Those familiar with corn ethanol costs will quickly notice that feedstock costs are a much smaller percentage of the total cost than can be expected for a mature commodity-based

industry, which suggests more needs to be done to lower operating costs. Secondly, costs are fairly evenly distributed among feedstock, pretreatment, enzymes, saccharification and fermentation, and other unit operations including the boiler for burning the lignin. As no single cost dominates the process, future cost improvements will depend upon integrated approaches for global process savings.

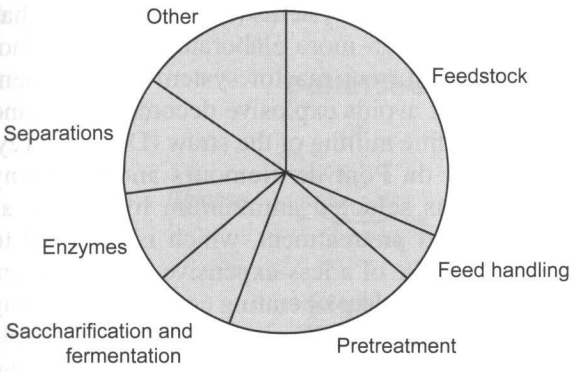


**Figure 4.** Cost contributions for each process area for converting corn stover to ethanol using dilute acid followed by SSF (adapted from Aden *et al.*, 2002).

The authors wish to emphasise the above scheme is only one of many proposed for converting biomass to ethanol. All processes, however, share the same operations: pretreatment, enzymatic hydrolysis, inhibitor abatement and fermentation. In the remainder of this review, each of these unit operations will be discussed in more detail.

## PRETREATMENT OF BIOMASS

The goal of pretreatment is simple in concept: to open up the structure of the cell wall to enable enzymes to convert cellulose to glucose. The success of pretreatment is easily measured by treating the washed solids with cellulase in a dilute solution and measuring release of glucose (Brown and Torget, 1996; Zhang *et al.*, 2007). Yet, despite this simple operational definition, there is no universal theory on what makes pretreated biomass highly digestible. A number of traits have been strongly correlated with digestibility for pretreated biomass (Dien *et al.*, 2005; Himmel *et al.*, 2007). For the most part, these directly relate to allowing the cellulase enzymes ready access to the individual cellulose strands. They include reduced particle size and increased porosity, removal of the surrounding hemicellulose, displacement or removal of the lignin away from the cellulose fibres, swelling of the microcrystalline cellulose fibres and breaking apart individual glucan strands to generate more ends. This is just a partial list of the most readily identifiable factors. Most pretreatments do not achieve all of these goals and those that do often use multiple mechanisms.

Pretreatments rely on a variety of chemical mechanisms to achieve the aforementioned goals. Hemicellulose can be solubilised in alkali or partially hydrolysed in dilute acid. Water alone can also act directly as a weak acid when heated to high temperatures (e.g. greater than 180°C), which increases its dissociation constant (Allen *et al.*, 2001). Organic acids released from the xylan continue to raise the acidity of the water. On the other hand, employing a mineral acid catalyst has the important advantage, compared to other pretreatments, of saccharifying xylan to monosaccharides, which readies them for fermentation.

Lignin is much harder to transform because of its strong ether bonds. The ether bonds can be broken by oxygen radicals, which can be introduced by treating biomass with ozone or hydrogen peroxide or via wet oxidation. Wet oxidation consists of pretreating the wetted biomass at high temperatures under a pressurised oxygen atmosphere (McGinnis *et al.*, 1983; Bjerre *et al.*, 1996). Alternately, lignin will begin to reform when heated above its glass transition temperature of 130–160°C (Hatakeyama *et al.*, 1982) and will be partially solubilised when also exposed to acids. Finally, in the special case of warm-season grasses, ester bonds formed between ferulic acid and arabinose join the lignin and hemicellulose together. These bonds can be directly saponified by treating under alkaline conditions.

Cellulose microfibrils are held together by a tight network of hydrogen bonds that form within individual glucan strands and between adjacent strands (Zhang and Lynd, 2006). The strands are quite crystalline and exclude water and enzymes from binding. Pretreatment of cellulose breaks apart these bonds and swells the fibres. Hydrogen bonds can be either broken thermally or by using solvents that interfere with hydrogen bonding. Examples of the latter are strong acid (concentrated $H_3PO_4$, HCl, or $H_2SO_4$) and (specific) room temperature ionic solutions that act as solvents for cellulose.

Most pretreatments encompass multiple mechanisms. Consider the dilute acid pretreatment described earlier. The mineral acid catalyses the hydrolysis of the hemicellulose, partially breaks apart and 'melts' the lignin, swells the cellulose and increases porosity while steam explosion reduces particle size (Saddler *et al.*, 1993; Holtzapple *et al.*, 1989). Another popular pretreatment is alkaline hydrogen peroxide. Here, the alkaline catalyst solubilises the xylan, saponifies ester bonds and disrupts the cellulose hydrogen bonding, while the peroxide ions disrupt the lignin network by breaking apart ether bonds (Gould and Freer, 1984; Gould, 1985b). It is important to remember that pretreatment conditions, and often even the type of pretreatment, need to be tailored to the source of biomass. For example, alkaline peroxide is highly effective against grasses (up to 100% cellulose enzymatic conversion to glucose) but not against more recalcitrant woody materials (approximately 50% conversion) (Gould, 1984).

In 2000, the major US laboratories that research pretreatment processes organised a consortium (Biomass Refining Consortium for Applied Fundamental Innovations, CAFI) to coordinate their efforts. This group first selected corn stover as a target substrate. The technologies evaluated were flow-through dilute acid, AFEX or ammonia fibre expansion (formally explosion), liquid hot water and high-pressure liquid ammonia percolation. This group jointly published their results on corn stover in a special journal issue that can serve as a useful guide in evaluating the state-of-the-art in pretreatment technology (Lloyd and Wyman, 2005; Wyman *et al.*, 2005a; Wyman *et al.*, 2005b; Kim and Holtzapple, 2005; Mosier *et al.*, 2005a; Teymouri *et al.*, 2005; Liu and Wyman, 2005; Tae and Lee, 2005; Eggeman and Elander, 2005; Mosier *et al.*, 2005b). Other pretreatments worth mentioning include alkaline peroxide (Gould, 1984; Gould and Freer, 1984; Gould, 1985a; Gould, 1985b), organosolv (Mosier *et al.*, 2005b; Pan *et al.*, 2006; Chum *et al.*, 1988; Asiz and Sarkanen, 1989), ozone (Miron *et al.*, 1981; Neely, 1984), and, most recently, room temperature ionic solution (Fort *et al.*, 2007; Dadi *et al.*, 2006). Further pretreatments for herbaceous biomass are also described in a recent review by this author (Dien *et al.*, 2005) and by more general reviews (Mosier *et al.*, 2005b; Sun and Cheng, 2002).

It is important to realise that high-fibre streams require their own special process considerations and reactor designs in comparison to process streams typically dealt with in current ethanol plants. The cost and complexity of the reactor are directly correlated to the severity of the pretreatment. So, for example, steam explosion with or without an added acid catalyst and high-pressure ammonia pretreatment would probably favour a continuous steam-explosion-type reactor such as originally developed for pulping wood (for a schematic see SunOpta BioProcess Inc., 2007). Integrated Biomass Utilisation Systems (IBUS, Denmark) has developed an even more elaborate three-stage hot water countercurrent reactor system for treatment of straws that avoids explosive decompression and the need for fine milling of the straw (Dong Energy, 2006). E. I. du Pont de Nemours and Company (DuPont) has selected ammonium hydroxide as their preferred pretreatment, which is expected to allow for the use of a less-expensive reactor design because of the milder operating conditions. Among the simplest are the PVC pipes used by Holtzapple and colleagues for lime pretreatments (Kim and Holtzapple, 2005). Unfortunately, downstream costs for recovering the lime likely negate reactor savings in this case (Eggeman and Elander, 2005). Forage treatments have also developed numerous inexpensive treatment systems suitable for farmers to apply (e.g. piles of biomass covered with plastic for ammonisation); however, these were centred around increasing digestibility for livestock feeding and were not designed for the higher sugar yields required for bioethanol (Sundstol and Owen, 1984).

## INHIBITOR MITIGATION

In the process of breaking down the cell wall structure, compounds are released that are detrimental to subsequent fermentation. These chemicals can include salts from neutralisation of the mineral acid or base catalyst, furan compounds, weak organic acids and phenolics (Figure 5). For a comprehensive review see Almeida *et al.*, 2007. Even when biomass is treated with acid or base, excess salts may be eliminated. For example, sulphuric acid is usually neutralised with lime, which forms gypsum, and ammonia can be evaporated and recycled. The degree to which organic acids and lignin aromatics are problematic varies with the pretreatment conditions and source of feedstock. Organic acids originate from the hemicellulose (e.g. acetate and ferulate)

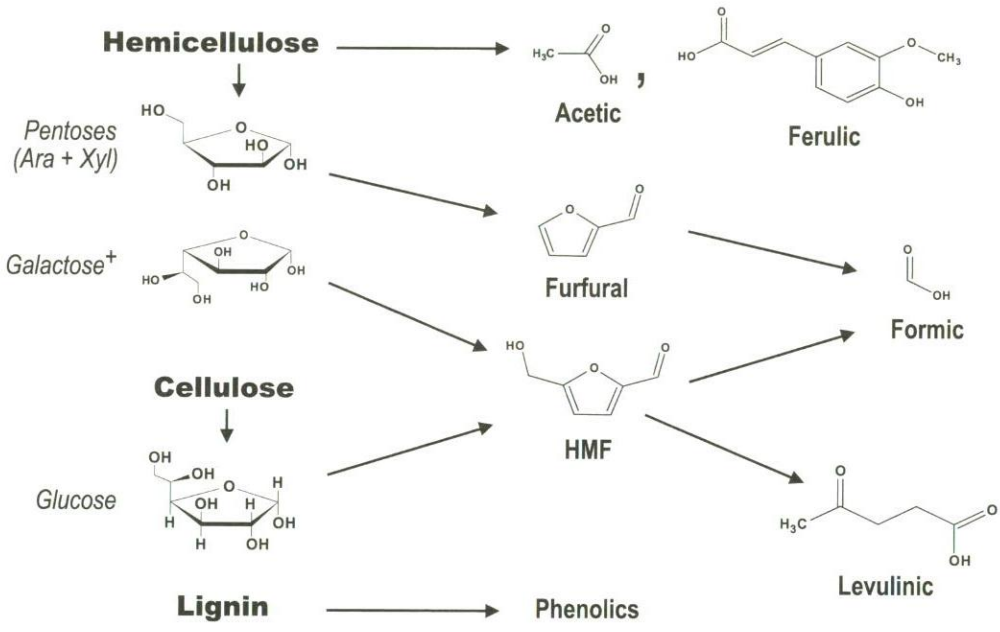


**Figure 5.** Many side-products of pretreatment will inhibit fermentation, even when present at low concentrations. These include organic acids released from hemicellulose, furans (furfural and HMF) produced from sugar decomposition and phenolics from lignin breakdown.

or sugar degradation. Acetic acid tends to be the most cited of the organic acids because it occurs at higher concentrations than the others. Of course, the toxicity of acetic acid varies with pH, and one way to minimise its impact is to ferment at higher pH values. Along with acetic acid, furans are probably the most troublesome and most studied of the inhibitors (Zaldivar *et al.*, 1999; Boyer *et al.*, 1992; Navarro, 1994). Once glucose, xylose and other sugars are released under harsh acidic pretreatment conditions, they can undergo further reactions to form hydroxy-methyl-furfural (HMF), or furfural. Both of these aldehyde compounds are quite toxic to microbes even when present at low concentrations (ibid). As might be suspected, higher concentrations of furfural are commonly observed with dilute acid than other pretreatments, because this pretreatment converts xylan directly to monosaccharides. The presence of inhibitors needs to be viewed holistically, meaning that they act in concert on the cells (Zaldivar and Ingram, 1999; Zaldivar *et al.*, 1999).

There are several strategies for dealing with these by-products. One of the oldest and still most popular is overliming, which consists of incubating the hydrolysate at an elevated temperature after adjusting the pH to 10 with lime (Leonard and Hajny, 1945; Martinez *et al.*, 2000; Mohagheghi *et al.*, 2006; Olsson *et al.*, 1995). This method reduces the amounts of furfural and HMF and has numerous other beneficial effects. However, it also leaves the process streams with high concentrations of gypsum ($CaSO_4$) and $Ca^{+2}$, which will cause problems downstream and represent a significant waste stream. Other methods reported include absorption (Frazer and McCaskey, 1989; Weil *et al.*, 2002), ion exchange (Frazer and McCaskey, 1989; De Mancilha and Karim, 2003), solvent-solvent extraction (Cruz *et al.*, 1999; Frazer and McCaskey, 1989) and biochemical and biological processing (Jonsson *et al.*, 1998; Larsson *et al.*, 1999; Nichols *et al.*, 2005). It should also be remembered that pretreatments that involve packed columns require solids- (e.g. fibre-) free streams to prevent plugging.

Alternatives to removing the inhibitors are to adapt the biocatalyst to grow in the hydrolysate (Liu *et al.*, 2005; Yomano *et al.*, 1998), to increase the beginning titre of cells (Chung and Lee, 1985) because microbes will reduce the aldehyde site on furfural and HMF to the less reactive alcohol form (Liu *et al.*, 2004; Villa *et al.*, 1992), to run fermentations in a fed-batch mode and to dilute out the inhibitory chemicals. Dilution has the shortcoming of diluting the substrate and

ultimately the final ethanol concentration. A newer method being pursued is to isolate genes related to furfural reduction and/or stress tolerance and to overexpress these genes in the biocatalyst (Petersson *et al.,* 2006; Gorsich *et al.*, 2006; Liu *et al.,* 2005).

## ENZYMES FOR BIOMASS CONVERSION

The relevant commercial enzymes for biomass conversion are cellulase, pectinase, hemicellulase and ligninases. However, commercial enzyme preparations are usually not pure and contain a wide variety of unreported activities. For example, preparations marketed as cellulases generally contain considerable hemicellulase activity (Dien *et al.,* 2007; Hespell *et al.,* 1997).

Cellulases are by far the most important of these because of their role in converting the abundant amounts of cellulose present in biomass to glucose. Three separate glycosyl hydrolase activities are needed for complete degradation of cellulose (Figure 6). Endoglucanases (EG, EC 3.2.1.4) hydrolyse internal glycosidic bonds primarily within amorphous regions. Exoglucanases or cellobiohydrolases (CBH, EC 3.2.1.19) bind at the ends of strands and travel down the attached strand progressively, primarily releasing cellobiose. Cellobiohydrolyases are directional and separate enzymes progress from the reducing and nonreducing ends. Finally, β-glucosidase (BGL, EC 3.2.1.21) completes the process, saccharifying the cellodextrins and cellobiose to glucose (for an excellent review see Zhang and Lynd, 2004).

The fungus *Trichoderma reesei* is commonly used for production of commercial cellulases for biomass conversion (Nieves *et al.*, 1998). *T. reesei* produces five EG enzymes, two CBH (one reducing CBH1 and one nonreducing enzyme CBH2), and two BGL enzymes (Vinzant *et al.*, 2001). CBH enzymes are critical for hydrolysis of crystalline cellulose and account for more than 60% of the total secreted protein produced when induced for production of cellulases. *T. reesei* cellulases have optimal activities at pH 4.5–5.0 and at 55–60°C. Unfortunately, cellulases are very sensitive to end-product inhibition. Excess glucose formation will inactivate BGL, and this in turn leads to accumulation of cellobiose, which inhibits EG and CBH. Depending upon the commercial source of cellulase, additional BGL may be needed to alleviate end-product inhibition. On a practical note, it is commonly realised that adding surfactants like Tween 80 to hydrolysates either before or after pretreatment improves glucose yields at lower cellulase loadings, presumably by preventing adsorption of the cellulases to lignin

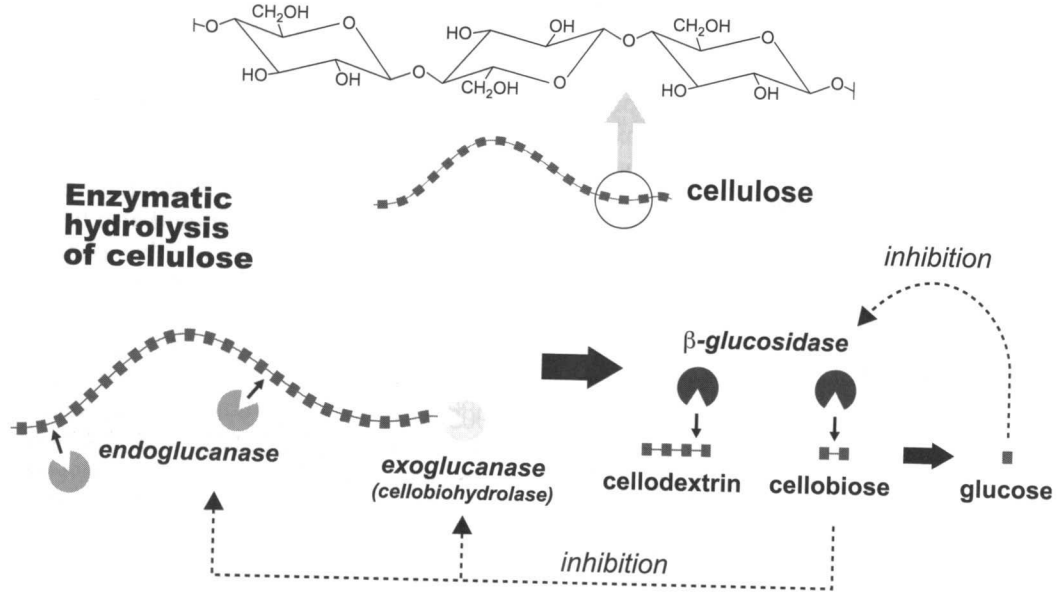


**Figure 6.** Schematic of cellulose hydrolysis by *T. reesei* enzymes (adapted from a diagram by Chris Skory, NCAUR, Peoria IL).

(Kristensen *et al.*, 2007; Wu and Lu-Kwang, 1998; Kurakake *et al.*, 1994).

Hemicellulase enzymes play two roles in bioconversion. First, all pretreatments other than those using mineral acid catalysts only partially hydrolyse xylan, and most likely hemicellulases will be needed to complete saccharification. There is no commercial hemicellulase marketed for complete xylan hydrolysis; hemicellulases are currently marketed for applications that only require partial xylan hydrolysis. Second, xylan removal is strongly correlated to cellulose digestibility. Therefore, it might be possible to compensate for lowering the severity of the pretreatment by adding additional xylanases. This has been determined to be the case for a variety of steam-treated biomasses (Berlin *et al.*, 2007; Berlin *et al.*, 2006; Berlin *et al.*, 2005).

Developing efficient hemicellulases for biomass conversion is likely to be challenging because the preparations need to be literally as complex as hemicellulose. Each unique chemical bond requires its own enzyme for hydrolysis (Figure 7) (Saha, 2003). As discussed earlier, hemicelluloses originating from grasses have a xylose backbone with much of the xylans substituted with short chains consisting of neutral and acidic sugars and organic acids. The sugar groups are primarily attached to carbon 4 of the xylose unit, but can be attached to carbon 3 as well. The enzymes needed to break down unsubstituted xylan are endoxylanase (1,4-β-D-xylan xylanohydrolases, EC 3.2.1.8) and β-xylosidase (1,4-β-D-xyloside xylohydrolase, EC 3.2.1.37). Endoxylanase splits apart xylan strands and β-xylosidase hydrolyses the shortened chains (X2-X5) to xylose. Additional enzymes are required for hydrolysis of xylan because side-chain substitutions on the xylan backbone protect it from these enzymes and therefore must also be removed (Saha, 2003). Side chains include neutral sugars, organic acids (linked by ester bonds) and uronic acids. As indicated earlier, commercial cellulases contain significant hemicellulosic enzyme activities, with the notable exception of feruloyl esterase (Dien *et al.*, 2007).

Pectinases are used to digest pectin, a linear chain of α-(1-4)-linked D-galacturonic acid that forms the pectin backbone. Like xylan, pectins also have multiple side-groups. We have determined that commercial pectinases are valuable for hydrolysing corn pericarp xylan, suggesting that pectinase preparations may have application beyond hydrolysing pectin (Dien *et al.*, 2007; Wu and Lu-Kwang, 1998).

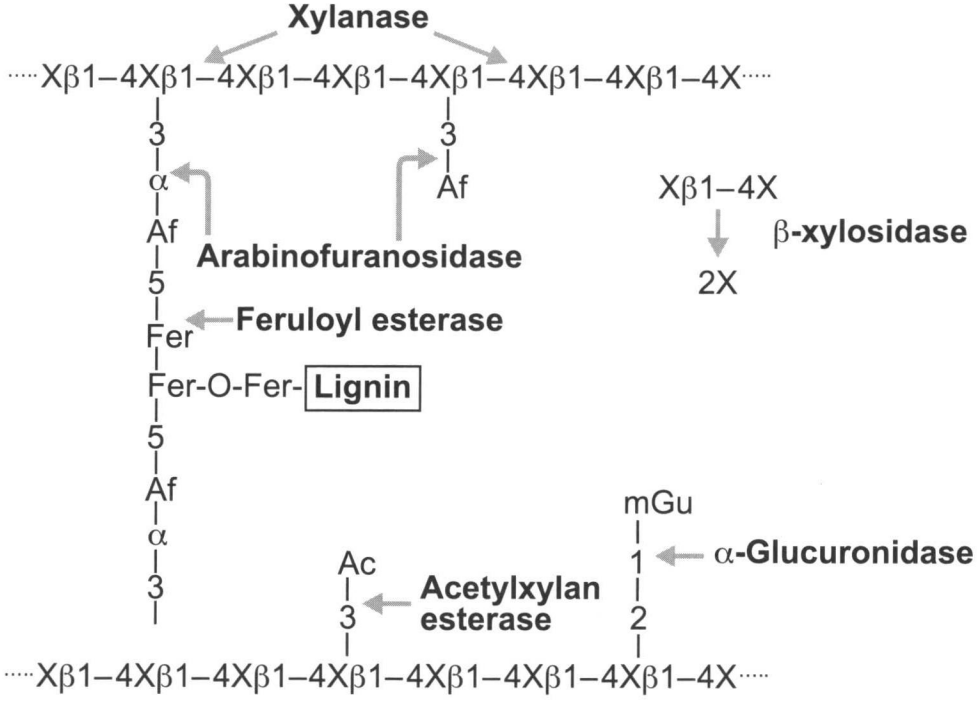


**Figure 7.** Hydrolysing xylan requires many separate enzymes because of its complex structure (adapted from Selinger *et al.*, 1996).

Ligninases include lignin peroxidase, manganese peroxidase and laccase. These enzymes have not been widely applied to pretreatment except indirectly in biological pretreatments. Laccases have been found to slightly increase the efficiency of cellulases and to aid in detoxifying hydrolysates, presumably by condensing lignin-related aromatics.

Perhaps no one area of research has the potential to revolutionise biomass conversion as does work on enzymes. The announced DOE target cost for cellulase formulations is 10 to 15 cents per gallon (Himmel *et al.*, 1999). Current cost estimates, which have fallen quite a bit in recent years, vary from 20 to 50 cents per gallon (Moreira, 2005; Somerville, 2007), but these are just estimates, and the real cost will be highly dependent upon the feedstock and pretreatment. To understand the true importance of enzyme costs, it is instructive to compare the enzyme loadings for amylases and cellulases. In a recent study from Vijay Singh's laboratory, Wang *et al.* (2007) applied a granular starch amylase for SSF of ground corn to ethanol. They used a loading of 4.9 mg/g starch and reported a conversion efficiency of 83% in 48 hours at an average productivity of 2.8 g/L/h. In another recent study, Tucker *et al.* (2003) studied the conversion by SSF of pretreated corn stover into the ethanol. The corn stover was pretreated by steam explosion in the presence of dilute sulphuric acid, and only the washed solids, which contained most of the cellulose, were fermented to ethanol. They used a cellulase loading of approximately 278 mg/g cellulose (assuming the cellulase preparation contained approximately 90 filter paper units, or FPU, per gram – Nieves *et al.*, 1998), and they reported a conversion efficiency of 90% in about 72 hours at an average productivity of 0.42 g/L/h (estimated from Figure 3). Directly comparing the results, the stover cellulose required over 50 times more enzyme preparation, and the resulting SSF was 6.7 times slower! The situation for cellulase is a bit better than it appears, since xylose was recovered at high efficiency (greater than one third of the total carbohydrate). Newer cellulases, developed by the two enzyme companies under a US federally funded research program, have reportedly improved activities compared to what was used in this study.

## FERMENTATION OF HYDROLYSATES

Only two known microorganisms, the yeast *Saccharomyces cerevisiae* and the bacterium *Zymomonas mobilis*, are considered suitable for commercial ethanol production. Both have exceptional ethanol tolerance (greater than 15% v/v), yields (more than 90% of theoretical), and high productivities (more than 2.5 g/L/h). Of these two, *S. cerevisiae* is preferred because it is more robust in industrial fermentations and less prone to contamination by opportunistic bacteria. In addition, yeast can be purchased in active dry form with high viability and can be stored for up to two years. However, neither microbe ferments xylose and, as previously mentioned, xylose represents more than 30% of the carbohydrates found in herbaceous plants and hardwoods (Dien *et al.*, 2003).

There are a few yeasts that are able to ferment xylose to ethanol with significant yields (Du Preez and van der Walt, 1983; Slininger *et al.*, 1982; Slininger *et al.*, 2006). Commercial interest has lagged because of the inability of these strains to grow anaerobically on pentoses and their low specific productivities. This has led proponents of these strains to apply recombinant techniques in a continuing quest to develop industrially suitable strains (Jeffries *et al.*, 2004 and 2007). Most molecular microbiologists, however, have turned to other platform microorganisms for engineering strains that will convert biomass sugars to ethanol. Two approaches have been undertaken. The first is to construct *Z. mobilis* (Deanda *et al.*, 1996; Mohagheghi *et al.*, 2002; Zhang *et al.*, 1995) and *S. cerevisiae* strains to metabolise xylose (and sometimes the other predominant pentose, L-arabinose). Xylose metabolism has been introduced into *S. cerevisiae* by borrowing the pathway from native xylose-fermenting yeasts (Den Haan *et al.*, 2007a; Den Haan *et al.*, 2007b; Fujita *et al.*, 2004; Ho *et al.*, 1998; Sedlak and Ho, 2004; Wahlbom *et al.*, 2003) or more recently by introducing a functional xylose isomerase (Kuyper *et al.*, 2004; Kuyper *et al.*, 2005). Research on *Saccharomyces* is particularly intense and is being pursued in laboratories all over the world. The other approach is to use bacteria that normally ferment xylose and other sugars and create strains that selectively produce ethanol. Specifically, this has meant expressing the two terminal enzymes in ethanol production from *Z. mobilis* and eliminating genes responsible for production of other fermentation products. Microorganisms successfully engineered with the second approach include the gram-negative bacterium *E. coli* (Ingram *et al.*, 1987; Hespell *et al.*, 1996; Ohta *et al.*, 1991a; Yomano *et al.*, 1998) and

*K. oxytoca* (Ohta *et al.*, 1991b; Wood and Ingram, 1992; Wood *et al.*, 2005). Strains representing all four of these species are being pursued for commercialisation, and research is still continuing on construction of superior versions of each (Table 2).

**Table 2. Commercialisation efforts for selected microorganisms.**

| *Host microbe* | *Inventor* | *Licensee* |
|---|---|---|
| *E. coli* and *K. oxytoca* | LO Ingram (U. of Florida) | Verenium Corp. (Cambridge, MA) |
| *Z. mobilis* | M Zhang (NREL) | DuPont (Wilmington, DE) |
| *S. cerevisiae* (XRH/XDH)[1] | N Ho (Purdue U.) | Iogen Corp. (Ottawa, ON, Canada) |
| *S. cerevisiae* (XI)[2] | JT Pronk (Delft/Royal Nedalco) | Mascoma Corp. (Cambridge, MA) |

[1] *P. stipitis* genes (xylose reductase and xylitol dehydrogenase) used for metabolism of xylose.
[2] Xylose isomerase used for metabolism of xylose.

In addition to the above-mentioned species, other species are being developed for ethanol production. The more important ones include mesophilic gram-positive bacteria, thermophilic gram-positive bacteria and *Corynebacterium*. Efforts at engineering *Bacillus* spp. and lactic acid bacteria to selectively produce ethanol have been largely unsuccessful to date, perhaps in part to problems in expressing pyruvate decarboxylase. Thermophilic gram-positive bacteria are considered potential hosts for CBP (Lynd *et al.*, 2005). *Clostridium thermocellum* is favoured for fermentation of cellulose, but it does not ferment pentoses. Potential pentose-fermenting microbes that can be teamed with *C. thermocellum* include either other *Clostridium* or *Thermoanaerobacter* species (Demain *et al.*, 2005). The most recent advance in the field is the targeted deletion of genes in *T. saccarolyticum*, which eliminated acetate and lactate production (Desai *et al.*, 2004). A significant finding in this regard is that pyruvate decarboxylase activity is not necessary to convert glucose solely to ethanol – a lesson echoed in a recent paper from Ingram's laboratory on *E. coli* (Kim *et al.*, 2007). Finally, *Corynebacterium* is a strict aerobe. The strategy behind applying this microorganism is to harvest it from aerobic cultures and essentially use it as a 'bag of enzymes' for converting sugars into ethanol. Extremely high productivities of up to 30 g/L/h have been reported on glucose (Inui *et al.*, 2004), and early progress is being made at introducing the genes needed for xylose metabolism (Kawaguchi *et al.*, 2006). However, further progress is needed to eliminate succinate production in the highest producing strain as well as the need for an external supply of pyruvate.

Giant strides have been made in recent years in developing biocatalysts that are commercially relevant. However, further improvements are still needed to increase xylose fermentation rates and tolerance to inhibitors. In a corn ethanol plant, more than 100 grams of ethanol per litre is produced within 40 hours with a conversion efficiency of 90%. Goals for corn stover, as outlined by DOE, are more modest at about 60 g/L ethanol produced in 72 hours with a conversion efficiency of 85% based upon all carbohydrates (US Department of Energy, 2006). Recent examples for laboratory-scaled biomass fermentations to ethanol are presented in Table 3. Yields are reasonable compared to those realised in starch conversion, except for the fed-batch SSF, in which the whole pretreated slurry was fermented without overlimiting. In general, the bacteria used had higher specific productivities than the *Saccharomyces* yeasts, and all rates were well below those considered normal for the corn ethanol industry. Maximum ethanol concentrations are also much lower than those routinely obtained from fermentation of corn starch. Finally, there is the case of *E. coli* that ferments at a neutral pH that is not compatible with cellulases from *T. reesei* and, therefore, cellulose will need to be fermented in a different bioreactor or completely saccharified prior to fermentation.

One point not made frequently enough is that from a process perspective, ethanol yield, productivity and maximum concentration are all interrelated. While researchers typically end fermentation either after sugars are exhausted or fermentation has stalled, commercial fermentations will be ended when slowing productivity makes the incremental cost of the additional ethanol too expensive. In other words, gains in productivities can be traded for reductions in yields and titres. There are two caveats, before moving on, concerning Table 3. Each listing was done under very different conditions and, therefore, it is difficult to compare specific organisms from this data, and for some organisms more recent data with improved results have been withheld for commercial considerations.

**Table 3. Sample rates and yields for converting herbaceous biomass to ethanol.**

| *Biocatalyst* | *Feedstock* | *Pretreatment* | *Max. ethanol (g/L)* | *Efficiency*[1] *(% theory)* | *Vp*[1] *(g/L/h)* |
|---|---|---|---|---|---|
| *E. coli* FBR5[2] | Wheat straw | Alkaline $H_2O_2$ | 19 | 80 | 0.39 |
| *E. coli* LY01[3] | Sugar cane bagasse | Dilute acid hydrolysate | 36 | 90 | 0.75 |
| *Z. mobilis* 8b[4] | Corn stover | Steam explosion | 54 | 91 | 1.1 |
| *Saccharomyces* TMB3400[5] | Corn stover | Steam explosion | 37 | 59 | 0.38 |
| *Saccharomyces* 424A(LNH-ST)[6] | Corn stover | Hot water | 23 | 88 | 0.42 |

[1] Efficiency is the percent of theoretical ethanol realised based upon initial sugars or carbohydrates. Vp is the average ethanol productivity in g ethanol per reactor volume per hour.

[2] Pretreated material was saccharified for 120 hours and inoculated with 5% v/v of a culture grown overnight (Saha and Cotta, 2006).

[3] Pretreated syrup was overlimed and inoculated 0.165 g DM/L. Statistics estimated from Figure 2 (Martinez *et al.*, 2000).

[4] Pretreated syrup was overlimed, diluted to 80% w/w and supplemented with glucose up to 100 g/L. Inoculum level was 0.06 g DM/L (Mohagheghi *et al.*, 2004).

[5] Pretreated material was diluted to 79% w/w and converted in a fed-batch SSF. Inoculum level was 5 g DM/L (Ohgren *et al.*, 2006).

[6] Pretreated material was saccharified for 96 hours and inoculated with 9 g DM/L (Mosier *et al.*, 2005a).

## SUMMARY

Industrial production of ethanol from fibrous biomass is right around the corner, as it has seemed for the past 50 years. But, today, two factors suggest that beyond the rhetoric, biochemical conversion of fibrous biomass will be shortly evaluated at commercial scale. The first is a confluence of events and geopolitical concerns that has convinced large corporations and institutional investors that investing in the technology to do so is worthwhile. Very few believe that oil will become inexpensive, that our sources will grow more secure, or , given the increasingly stringent warnings from climate scientists, that oil will continue to be burned with the same abandon as in the previous century. This trend has no doubt been aided by the investments and great profits from the corn ethanol industry and is made possible (as earlier described) by the willingness of the government to make vast investments in energy. The second factor is that, since the oil crisis of the 1970s, scientists have been quietly working away enhancing the technology to make it feasible, to lower technological risks, and to reduce operating costs. The ability of Iogen Corp. to successfully produce ethanol from straws in their 2.5 million-litre-per-year capacity demonstration plant is powerful evidence for this statement. Still, there is little doubt that investment in lignocellulosic ethanol is very risky, and investors are probably motivated at this point by the opportunities, if successful, of licensing the technology to others as opposed to profiting directly from the ethanol produced.

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